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14. ABSTRACT

Our preliminary data had suggested that HOXC5 gene expression was increased in prostate tumor and in tumor-associated normal epithelium compared to epithelium from normal prostate, raising hopes that HOXC5 could be a biomarker that could guide whether a patient with a negative biopsy should undergo a rebiopsy. We compared methods for preparation of RNA from formalin-fixed, paraffin embedded tissue and used the best method to prepare RNA from normal prostate tissue and from dissected tumor and tumor-associated normal tissue. While there was a trend toward increased frequency of HOXC5 expression in tumor and tumor-associated normal tissue compared to normal prostate, the trend fell slightly short of statistical significance. Moreover, there was only a small difference between the frequency of detection of HOXC5 expression in normal and tumor-associated normal. These results do not support the efficacy of HOXC5 as a biomarker for the presence of tumor elsewhere in the gland.

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INTRODUCTION

Published estimates have indicated that half of prostate biopsies that are judged histologically normal are in fact false negatives. That is, in half of the men, with negative biopsies the biopsy sampling has missed the tumor. As a result, these men are faced with undergoing a rebiopsy or a period of "watchful waiting." Our preliminary data in a limited set of samples had suggested that expression of the homeodomain gene, HOXC5, was expressed in prostate tumor and in normal epithelium from tumor-containing glands and expressed at much lower levels or not at all in normal epithelium from tumor-free prostates. Tumor-free prostate tissue is obtained from cystoprostatectomies, donors, and from surgeries for BPH, benign prostatic hyperplasia. The goal of this work was to develop technologies to prepare RNA from paraffin-embedded, formalin fixed tissue of sufficient quality to be used for biomarker detection, then to employ these methods to evaluate HOXC5 as a biomarker in normal prostate epithelium that would indicate the presence of tumor elsewhere in the gland.

BODY:

Two Tasks were listed in the approved Statement of Work:

Task one: To evaluate HOXC5 as a marker of cancer presence in normal, tumor-associated prostate.

Task two: to develop an assay for HOXC5 expression using formalin-fixed, paraffin embedded biopsy specimens.

At the time the proposal was written and reviewed, the preparation of usable RNA from formalin-fixed paraffin embedded biopsy specimens was a methodology that had only recently been reported to be successful. As we began to embark on these experiments more reports began to emerge that successfully recovered sufficiently intact RNA to use for detection and quantification of RNA. This however made us change strategies slightly. We had originally proposed to evaluate the Arcturus "Paradise" reagent System. This was the first system to our knowledge for preparation of usable RNA from formalin-fixed paraffin embedded clinical specimens. It was, however, inordinately expensive, prohibitively so for widespread clinical use. Since mulitiple independent reports suggested that other strategies could be used we saw no point in using the Arcturus system as it would not be cost effective for the hoped for clinical use. We did however compare 3 other methods for RNA preparation from formalin-fixed paraffin embedded tissue specimens, the Ambion FFPE RNA isolation kit, the Roches High Pure RNA paraffin kit, and a proteinase K/Trizol method.

To compare methods a 7 μm paraffin section was cut and deparaffinized on the slide. The area of interest was identified by comparing an unstained slide with a hematoxylin and eosin (H&E) stained adjacent section. The area of interest was dissected with a razor blade and transferred to a 1.5 ml microfuge tube. Two or three sections were cut depending on the size of the lesion. An example is shown in Fig. 1.

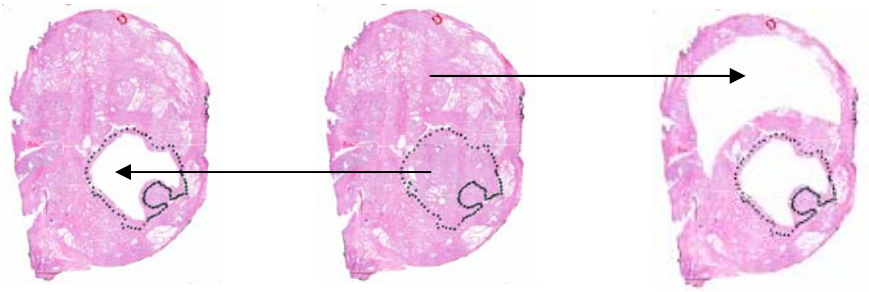

Fig 1. dissection of prostate sections for tumor and tumd tumor-asiated normal epithelium.

Method 1. Ambion FFPE RNA isolation kit. The tissue was digested with proteinase K in the buffer supplied with the kit. The protocol suggests 3 h or until tissue is liquefied. We found that 3 h was never long enough and so always digested overnight and the next day additional proteinase K was added for an additional 1-2 hours. The kit protocol then was followed for the remainder of the RNA isolation including DNAse I treatment. RNA was eluted from the last column in 20 μl.

Method 2. Roche High Pure RNA paraffin kit. The tissue was digested in the tissue lysis buffer, SDS, and proteinase K. The protocol indicated that digestion be at 55 C overnight but we upped that to 60 C since it seemed to make the digestion more effective. As in method 1, more proteinase K was added the next day and the digestion continued for 1 to 2 h. The kit protocol then was followed for the remainder of the RNA isolation including DNAse I treatment. RNA was eluted from the last column in 40 μl.

Method 3: Proteinase K/ Trizol. The dissected tissue was digested in 200 μl digestion buffer (20 mM Tris, pH 7.5), 20 mM EDTA, 1% SDS with 20 μl proteinase K (10 mg/ml). Incubation times of 2 h, 4h, 6 h, and overnight at 60-65 C were compared. As with the other methods, overnight digestion worked best. Isolation with Trizol-LS was performed according to the company protocol. RNA was treated with DNAse I then phenol/chloroform extracted, precipitated, and redissolved in 10-20 μl H_2O.

The amount of RNA isolated from this small amount of tissue is too low to quantify. It is also degraded as tested on an Agilent bioanalyzer. Therefore, the analysis of control genes is necessary to correct for RNA input. In these

studies we have employed the standard control of ribosomal RNA to make this correction. Samples were only used for further analysis if they had a Ct of 25 cycles or lower by Taqman analysis of ribosomal RNA.

For RT-PCR analysis of the RNA different RT enzymes, MMLV and superscript III, were tested. High input volumes of RNA from method 3 inhibited the reaction. For the other two methods, the maximum possible input of 10 µl per reaction worked fine. The use of superscript III reverse transcriptase resulted in the production of 4-16 times more cDNA. Methods 1 and 2 were both clearly superior to method 3. While method 1 sometimes resulted in higher yields, RNA prepared by method 2 gave more consistent results and yielded in a larger, more amenable final volume. For this reason further analyses were done using method 2. It was found that samples fixed and stored more than 5 years before RNA was prepared generally yielded RNA too degraded to be of use. More recent samples were usually, although not always, of acceptable quality. Typical results are presented in Figure one below. Three samples were prepared from adjacent tissue sections by the three methods described. Although method one appears to give a slightly higher yield (lower cycle number), only half as much RNA was used in the sample prepared method 2 since equal volumes (10 µl) of the final column eluate was used and method 2 gives twice as much eluate per sample.

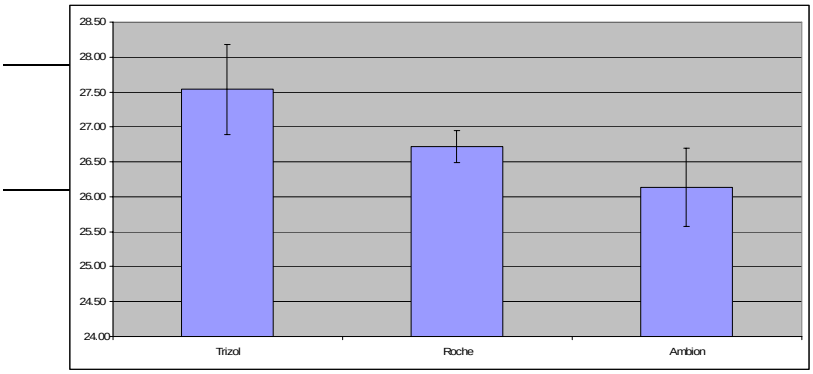

Fig. 2. Comparison of RNA preparation methods from formalin-fixed, paraffin embedded tissues. Y-axis is Taqman cycle number (Ct) for HOX C5. RNA prepared by method 3 (Trizol), method 2 (Roche), and method 1 (Ambion)

Analysis of HOXC5 expression in prostate tumor, tumor-associated normal, and normal prostate. RNA was prepared from formalin-fixed, paraffin embedded prostate tissue judged histologically as tumor, tumor-associated normal, or from tumor-free prostates derived from donors, from cystoprostatectomies, or from surgeries for benign prostatic hyperplasia. Taqman analysis was performed on the samples. Seventeen of the tumor/normal pairs were analyzed for the expression of markers closely associated with tumor, DD3 and AMACR, to ensure that proper dissection was accomplished. A value of ΔCt of greater than 10 was defined as negative expression for the gene of interest (GOI) and a ΔCt of less than 10 was defined as positive expression where $\Delta Ct = Ct_{GOI} - Ct_{ribosomal}$. Ct is the number of cycles to reach a Taqman signal of 50% of the plateau value. The data indicate that the dissected "tumor" samples reliably expressed the tumor markers and the "normal" from the same section do not.

These and additional cases were assessed for HOXC5 expression as was tissue harvested from prostates not bearing malignancies. The results for HOXC5 expression are shown in the following table:

	Pos	Neg
Tumor	14 (63.6%)	8 (36.36%)
Normal Tumor-associated	7 (41.18%	10 (58.82%
Normal prostate (presumably tumor free prostates)	3 (30.00%)	7 (70.00%)

Clearly, HOXC5 expression is not as robust a tumor marker as either DD3 or AMACR. There is, however, a trend for more detection of HOXC5 expression from normal, to tumor-associated normal, to tumor. In this still small sample set a Cochran-Armitage Trend Test yields a p value that falls just short of significance $p = 0.0573$. Disappointingly however, the difference in the frequency of detection of HOXC5 between normal prostate and tumor-associated normal was minimal. 41% of tumor-associated normal expressed HOXC5 and 30% of normal prostate had detectable expression. While there are explanations that one could posit as to why certain variables in the collection, definition, and sampling of human tissue could erode potential differences between normal and tumor-associated normal, in practice these data do not indicate that HOXC5 would be a robust marker. As the results are not entirely negative however, we intend to continue to assess for HOXC5 expression along with other markers as our number of samples available and technology of handling formalin-fixed paraffin embedded tissues progresses.

KEY RESEARCH ACCOMPLISHMENTS

- Development and optimization of technologies for preparing and analyzing RNA from formalin-fixed paraffin embedded prostate and prostate biopsies.
- Development of Taqman probes for detection of HOXC5 expression.
- Detection of HOXC5 expression in RNA prepared from formalin-fixed paraffin embedded clinical material.
- Initial evaluation on the utility of HOXC5 gene expression as a biomarker for distinguishing normal prostate from tumor-associated normal prostate.

REPORTABLE OUTCOMES – none

CONCLUSIONS

HOXC5 shows a trend toward increasing expression in tumor samples versus tumor-associated normal and normal prostate. This trend did not quite reach statistical significance on the current sample set. However, HOXC5 expression did not robustly distinguish tumor-associated normal prostate from prostate. Thus, at this point, the evidence does not support HOXC5 as a robust marker in normal prostate for the presence of tumor elsewhere in the gland.

REFERENCES – none

APPENDICES – none